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STUDIES OF GENETIC VARIATION IN THE AIDS VIRUS: RELEVANCE TO DISEASE PATHOGENESIS, ANTI-VIRAL THERAPY, AND VACCINE DEVELOPMENT

ANNUAL REPORT

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Genotypic variation among independent isolates of Human Immunodeficiency Virus Type 1 (HIV-1) is a widely recognized property of the virus [1-9]. The molecular characteristics and biological consequences of HIV-1 variation *in vivo*, however, are only beginning to be understood and this information is essential for elucidation of disease pathogenesis and vaccine development. This is a major objective of the work by Dr. George M. Shaw for Contract Number DAMD 17-87-C-7038. In the past year, we have examined the genesis of HIV-1 molecular variation in persistently infected individuals by preparing and analyzing recombinant HIV-1 lambda phage libraries from three virus isolates. Twenty-seven, 17, and 18 full-length HIV-1 clones were identified, mapped with 11 restriction endonucleases, and found to represent 17, 10, and 13 distinguishable but highly related viral genotypes, respectively. Recombinant viral clones from two HIV-1 isolates obtained from the same individual 16 months apart showed clear patterns of genotypic relatedness, yet progressive change. Similar changes in the HIV-1 genome did not occur *in vitro* during virus isolation and amplification. The results indicate that HIV-1 variation *in vivo* is rapid, that a remarkably large number of related but distinguishable genotypic variants evolve in parallel and coexist during chronic infection, and that "isolates" of HIV-1, unless molecularly or biologically cloned, generally consist of complex mixtures of genotypically-distinct viruses.

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In previous studies of HIV-1 variation, we observed that independent as well as sequential virus isolates from individual patients showed evidence of rapid genetic change[1, 2, 5, 8]. Genotypic analysis of paired HIV-1 isolates from donor-recipient transfusion cases[10] and nucleotide sequence comparisons of geographically distinct HIV-1 strains[4] provided further evidence for the highly variable nature of HIV-1. However, the spectrum of HIV-1 variation in chronically infected individuals, the mechanisms underlying this variation, and its biological consequences still remain unknown. To address this question, we have analyzed at a molecular level sequential virus isolates from two infected individuals (RJS and WMF) with HIV-1 associated clinical disease. Recombinant lambda phage libraries of one RJS isolate (RJS4) and two WMF isolates (WMF1 and WMF3) were prepared so that the viral DNA molecules which *in toto* comprise the hybridization patterns of the overall isolate DNA[8] could be dissected and analyzed individually. A total of 27, 17, and 18 full-length HIV-1 clones were obtained from the three libraries (Figure 1). Careful restriction enzyme mapping using 11 endonucleases revealed that 17 of the 27 RJS4 clones, 10 of the 17 WMF1 clones, and 13 of the 18 WMF3 clones were distinguishable by unique cleavage patterns. The remarkably large number of distinguishable viral clones within each isolate resulted from various combinations of restriction site polymorphisms distributed throughout the viral genomes. As expected, the restriction patterns of the predominant HIV-1 clones corresponded with the fragments visualized on blot-hybridizations of the isolate DNA from which they were cloned. Because each restriction enzyme generated only one to three different fragmentation patterns, the many unique viral genotypes shown in this report to comprise a virus isolate had previously gone unrecognized.

Inspection of the genomic restriction patterns of the HIV-1 clones in Figure 1 showed that clones from individual virus isolates (RJS4a-q; WMF1a-j; WMF1a-m) were considerably more similar to other clones within that isolate than to unrelated clones such as HXB2, LAV-Mal, LAV-Eli, ARV2, and WMJ1. In order to quantify objectively the extent of similarity among the different viral clones, a pairwise comparison of the percentage of restriction site differences between each of the 45 viral genotypes shown in Figure 1 was performed (total of 1,035 independent comparisons; Table 1). Such an analysis of restriction site differences between related clones is a valid means for estimating overall nucleotide sequence variability[11, 12]. Assuming that the loss of a restriction site in otherwise highly related genomes results from a change in a single nucleotide, a 50% difference in restriction sites when using enzymes that recognize 6 base pair sequences corresponds to approximately 8% nucleotide sequence difference (i.e., 1 nucleotide change out of every 12 nucleotides sampled). We used this approach first to analyze clones of HIV-1 for which nucleotide sequence information was available, and the correlation between nucleotide sequence differences and restriction site differences was found to be quite good. For example, clone HXB2 differs from clones BH10, ARV2, and LAV-Eli by 13%, 41%, and 62% in restriction sites (ref. 1 and Table 1). Based on these restriction site differences, the predicted nucleotide differences would be 2.2%, 6.8%, and 10.5%. The actual differences determined by nucleotide sequence comparisons[13] are 1.6%, 5.8%, and 9.7%, respectively. Similarly, clones of WMJ2 and WMJ3, both of which were derived from a single HIV-1 infected patient, differ by 12% in restriction sites and by approximately 1-2% in nucleotide sequence[8]. Clones of LAV-Mal, LAV-Eli, and ARV2 differ from each other by 58 - 70% in restriction sites (Table 1) and by 10.1 to 13.0% in nucleotide sequence[13]. In the present study, the restriction cleavage site differences among all 45 clones ranged from 3% to 70%. The viral DNA genomes comprising isolates RJS4, WMF1 and WMF3 were considerably more similar to other viral genomes from within the same isolate than to viral genomes from unrelated (independent) isolates. For example, the 17 different RJS4 genotypes varied from each other by 3 - 28% (mean of 13%) whereas the same clones varied from independent viruses by 41 - 70% (mean of 55%; $p<0.0001$). The 10 different WMF1 genotypes varied from each other by 3 - 33% (mean of 13%) and from independent viruses by 34 - 64% (mean of 54%; $p<0.0001$). The 13 different WMF3 genotypes varied from each other by 4 - 29% (mean of 13%) and from independent viruses by 38 - 65% (mean of

56%;$p < 0.0001$). These data show that while considerable genotypic diversity of viruses exists within a given isolate, these multiple distinguishable viral forms have all clearly evolved from one another, or from common precursor viruses, and do not represent concomitant infection by unrelated (independent) viruses.

Isolates WMF1 and WMF3 were derived from cultures of peripheral blood lymphocytes of the same individual taken 16 months apart. If the viral forms present in WMF3 had evolved sequentially from, or in parallel with, the viruses present in WMF1, the extent of similarity between WMF1 and WMF3 viral genomes should be intermediate between values for clones from within each isolate and clones from independent (unrelated) isolates. If superinfection with unrelated viruses had occurred, genotypes of WMF1 and WMF3 viruses would be expected to vary to the same extent as the unrelated viruses HXB2, LAV-Mal, LAV-Eli, ARV2, WMJ1, RJS4a, and WMF1a. The data in Table 1 show that the former, not the latter, was the case. The viral clones WMF1 (a-j) differed from clones WMF3 (a-m) by 11 - 44% (mean of 23%), an intermediate value statistically different from those for viruses from within single isolates (mean of 13%; $p < 0.0001$) or unrelated (independent) isolates (mean of 55%; $p < 0.0001$). These data, plus the unique similarities among WMF1 and WMF3 clones depicted in Figure 1, indicate that the viruses comprising the latter isolate evolved either from genomes present in the former isolate or from immediate precursor genomes common to both. The absence of clones in WMF3 that are identical to clones in WMF1, and the existence of certain viral genotypes in WMF1 and WMF3 that differ by as much as 39% in restriction sites (e.g., WMF1h and WMF3m), underscores the extremely rapid rate of change of HIV-1 *in vivo*.

As a control for these studies, and to determine the extent to which *in vitro* changes in HIV-1 could contribute to the observed genetic differences in RJS and WMF isolates, we determined the extent of HIV-1 genotypic variation occurring during cultivation and amplification of virus from infected patient tissues. Peripheral blood mononuclear cells (PBMC) obtained from patient RJS at the same time that isolate RJS4 had been obtained were divided into two parts and each was co-cultivated with PHA-stimulated PBMC from different HLA-unrelated normal donors. The restriction cleavage patterns of the two virus isolates obtained after 6 weeks of culture were identical (Figure 2A), implying that the virus populations isolated were representative of viruses present *in vivo*. PBMC taken from the same patient two and four weeks after the first phlebotomy yielded virus populations that were genetically related to the first isolates yet distinguishable (Figure 2B), reflecting the recognized genotypic heterogeneity of HIV-1 *in vivo*. Finally, the genotypic pattern of virus cultured from the brain of an HIV-1 infected patient (BB) was compared to viral DNA extracted directly from an *uncultured* specimen of the same tissue. The restriction patterns were identical (Figure 2C) indicating that the overall genotypic pattern of virus isolated by short-term culture corresponds to virus populations actually present *in vivo*. In a separate set of experiments, a recombinant lambda phage library identical to those prepared for RJS4, WMF1, and WMF3 was prepared from an isolate of HIV-1 that had been biologically-cloned by five sequential cell-free end-point dilutions of culture supernatant. This was done to obtain a genotypically-pure virus stock that could then be expanded in culture, as is done during the isolation of HIV-1 from human tissues, in order to determine how much genotypic variation is introduced during virus amplification in vitro. From this library, 10 full-length viral DNA clones were identified and these were identical in 270 out of 273 restriction sites mapped (average of 27 sites per clone; data not shown). This degree of restriction enzyme site variability (1%) in a biologically-cloned isolate that was amplified through multiple rounds of replication was significantly less than for the isolates RJS4 (13%), WPF1 (13%), and WPF3 (13%) (Table 1). These data thus suggest that the extensive genotypic variation identified within naturally-occurring isolates of HIV-1 described in the present study are not the result of changes occurring during *in vitro* virus propagation or molecular cloning, although it is recognized that under conditions of selective pressure significant genetic changes in the HIV-1 genome can and do occur *in vitro*[14].

The results of this study indicate that genotypic variation of HIV-1 *in vivo* is rapid and extensive, that numerous variant viral forms coexist over time within the same patient, and that

"isolates" of HIV-1 actually consist of complex mixtures of genotypically-distinct, albeit related, viruses. The present study also indicates that during natural infection by HIV-1, different viral genomes evolve in *parallel* and result in the emergence and persistence of multiple distinct genotypic forms (Figure 1, Table 1, ref. 8). The possible molecular mechanisms underlying this extensive genetic variability of HIV-1 have been discussed[4-9, 15, 16].

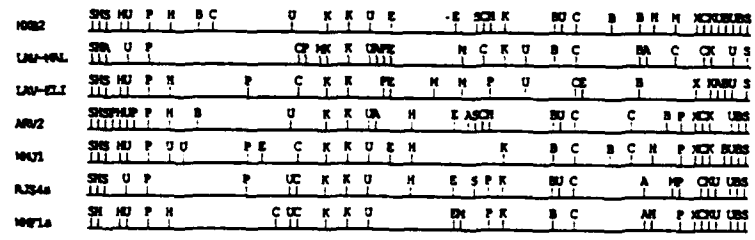
Variation of HIV-1 has parallels in other lentiviral systems including equine infectious anemia virus (EIAV), visna virus, and simian immunodeficiency virus (SIV)[17-19]. For EIAV, it is clear that genotypic changes are responsible for biologically important alterations in viral antigenicity which allow the virus to elude host immune defenses[20]. For visna, the significance of antigenic variation is less clear[19]. In the SIV system, a virus strain has recently been isolated from a pig-tailed macaque that possesses altered biologic and antigenic properties leading to a broader host-range and a rapid, fatal immunodeficiency syndrome several weeks after inoculation (P. Fultz, personal communication). There are indications that genotypic variation in HIV-1 is similarly associated with potentially important biologic differences among variant forms. We have examined the biologic properties of viruses derived by transfection of full-length WMF1.16 and WMF3.3 clones (patterns WMF1a and WMF3c in Figure 1) and found them to be indistinguishable from wild-type virus in T-cell tropism and cytopathicity (data not shown). We have also examined the biologic properties of hybrid viruses constructed by exchanging the envelope region of six different RJS4 clones (numbers 6, 15, 16, 22, 24, and 26) into the transfection-competent HIV-1 prototype clone HXB-2 (ref. 21) and have found that the progeny viruses exhibit striking biological differences. These findings are especially intriguing in light of recent findings by Mullins and co-workers who showed that the envelope/LTR region of a replication-defective variant of feline leukemia virus (FeLV), when introduced into a replication competent construct of FeLV, was responsible for inducing a highly reproducible fatal immunodeficiency illness in cats[22]. Furthermore, Gartner and Popovic have shown that some isolates of HIV-1 preferentially replicate in mononuclear phagocytes whereas others show preference for T-lymphocytes[23], and Koyanagi and Chen have identified genotypically-distinct mononuclear phagocytes and brain glioma explant cultures[24]. The results presented in this paper demonstrate the extreme plasticity of the HIV-1 genome as a genetic substrate for important biological variation. Finally, the observation that HIV-1 "isolates" generally consist of complex mixtures of genotypically-distinct viruses is in keeping with the general concept of RNA viruses as "quasispecies"[25, 26]. Experiments examining the genetic, biologic, and immunologic properties of HIV-1 will need to account for this extensive genotypic heterogeneity, and in some instances may require the use of biologically or molecularly cloned viruses.

The results of the year 01 studies summarized in this report represent important and critical progress toward all of the specific aims of the DAMD 17-87-C-7038 contract work. The molecular HIV-1 clones provide the genetic substrate essential to the completion of all objectives of this contract work. Parenthetically, I would add that we are in the process of depositing all of these molecular HIV-1 clones in the NIH AIDS repository so they will be available to members of the scientific community worldwide. Our contact at the AIDS Repository is:

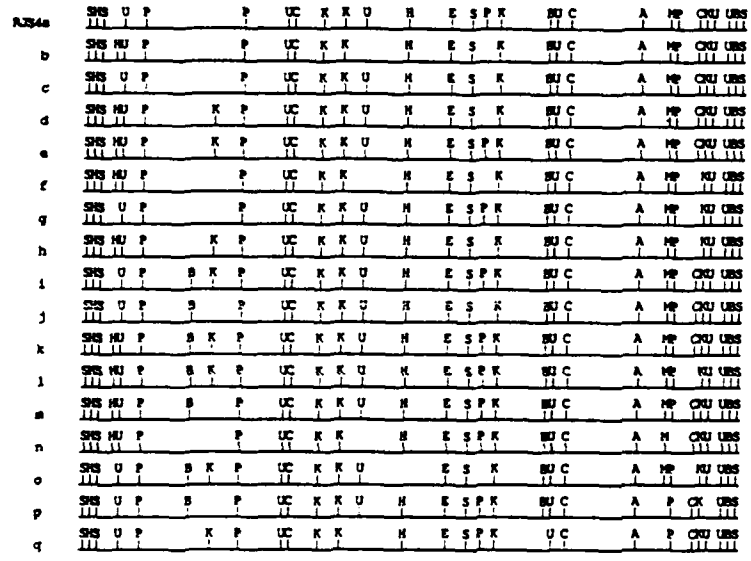
Dr. Steve Lindenfelser
NIAID AIDS Reagent Program
649A Lofstrand Lane
Rockville, MD 20850

Figure 1. Restriction endonuclease cleavage patterns for 7 independent HIV-1 viral clones (HXB2; LAV-Mal; LAV-Eli; ARV2; WMJ1; RJS4a; WMF1a) and for 17 distinguishable clones from isolate RJS4, 10 different clones from isolate WMF1, and 13 distinct clones from isolate WMF3. To the right of each set of restriction maps is a table summarizing the different restriction enzyme patterns for each representative clone within that set (E-EcoRI; S-SstI; X-XhoI; B-BglII; U-PvuII; H-HindIII; P-PstI; K-KpnI; M-BamHI; C-CvnI; A-XbaI). The top clone pattern in each set is arbitrarily designated 11111111111 and differences in subsequent clone patterns are identified sequentially. The laboratory clone (e.g., RJS4.1; RJS4.11; RJS4.16; etc.) designation and the total number of viral clones represented by each pattern are also indicated.

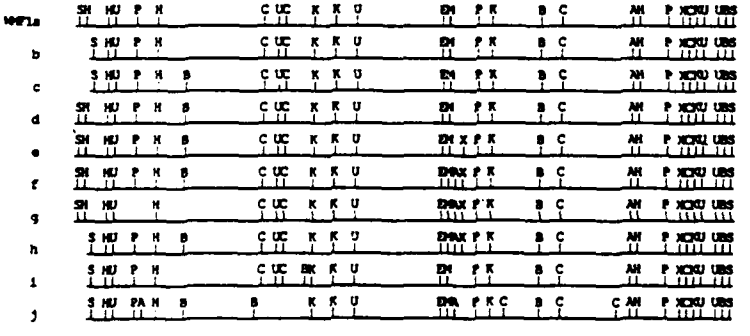
Methods: Non-amplified recombinant phage libraries were prepared in lambda-gt Wes/lambda B by standard techniques[1, 27]. Isolates of RJS4, WMF1, and WMF3 were previously described[8] and were obtained by co-cultivation of patient PBMC with normal donor PBMC followed by transmission and short-term (2 - 4 weeks) amplification of virus in Jurkat (RJS) and H9 (WMF) cells. RJS4 was cloned in permuted form by digestion of circular DNA intermediate forms using EcoRI. WMF1 and WMF3 were cloned from integrated and unintegrated viral DNA in non-permuted form using SstI. Approximately 1 x 10^6 recombinant phage were screened with a ^{32}P-BH10i probe[1], and all full-length HIV-1 clones detected were analyzed. Clones were mapped side-by-side using Southern blot-hybridization, single, double, and triple enzyme digests, and full-length, 5', and 3' HIV-1 probes to ensure mapping accuracy, as described[1]. Clones comprising WMF1b, c, h, i, and j patterns are approximately 170 base pairs shorter than the other clones shown due to the presence of an additional polymorphic SstI site located in the 5' leader sequence of these viruses. The restriction maps of HXB2, LAV-Mal, LAV-Eli, ARV2, and WMJ1 were deduced directly from their nucleotide sequences[13] or from previous publications[8].



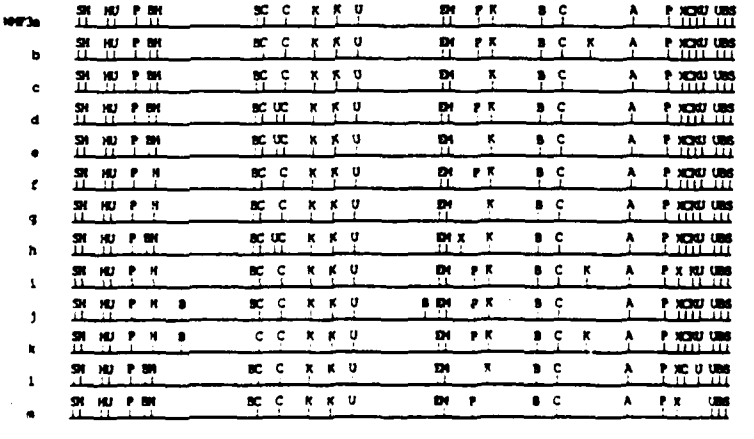
Virus	Pattern	
	E S X B U H P K M C A	
HXB2	1 1 1 1 1 1 1 1 1 1 1	
LAV-MAL	2 2 2 2 2 2 2 1 2 2 2	
LAV-ELI	3 3 1 3 3 3 3 2 3 3 3	
ARV2	4 1 1 4 4 4 4 2 4 4 4	
MAJ1	5 3 1 5 5 5 5 1 4 5 1	
RJS4a	4 1 2 6 1 6 6 1 1 6 5	
MNF1a	4 4 1 6 6 7 7 1 5 7 5	



Virus	Pattern	Clone	Total
RJS4	E S X B U H P K M C A		Number
a	1 1 1 1 1 1 1 1 1 1 1	1, 11, 16, 39, 46	5
b	1 1 1 1 2 2 2 1 1 1 1	2, 23, 26	3
c	1 1 1 1 1 1 2 1 1 1 1	18, 34	2
d	1 1 1 1 1 2 2 2 1 1 1	15, 40	2
e	1 1 1 1 1 2 1 2 1 1 1	20, 24	2
f	1 1 1 1 2 2 2 1 1 2 1	12, 31	2
g	1 1 1 1 1 1 1 1 1 2 1	7	1
h	1 1 1 1 1 2 2 2 1 2 1	32	1
i	1 1 1 2 1 1 1 2 1 1 1	42	1
j	1 1 1 2 1 1 2 1 1 1 1	6	1
k	1 1 1 2 1 2 1 2 1 1 1	22	1
l	1 1 1 2 1 2 1 2 1 2 1	14	1
m	1 1 1 2 1 2 1 1 1 1 1	21	1
n	1 1 1 1 2 2 3 1 1 1 1	45	1
o	1 1 1 2 1 3 2 2 1 2 1	19	1
p	1 1 1 2 3 1 1 1 2 1 1	43	1
q	1 1 1 3 2 1 1 2 2 1 1	4	1



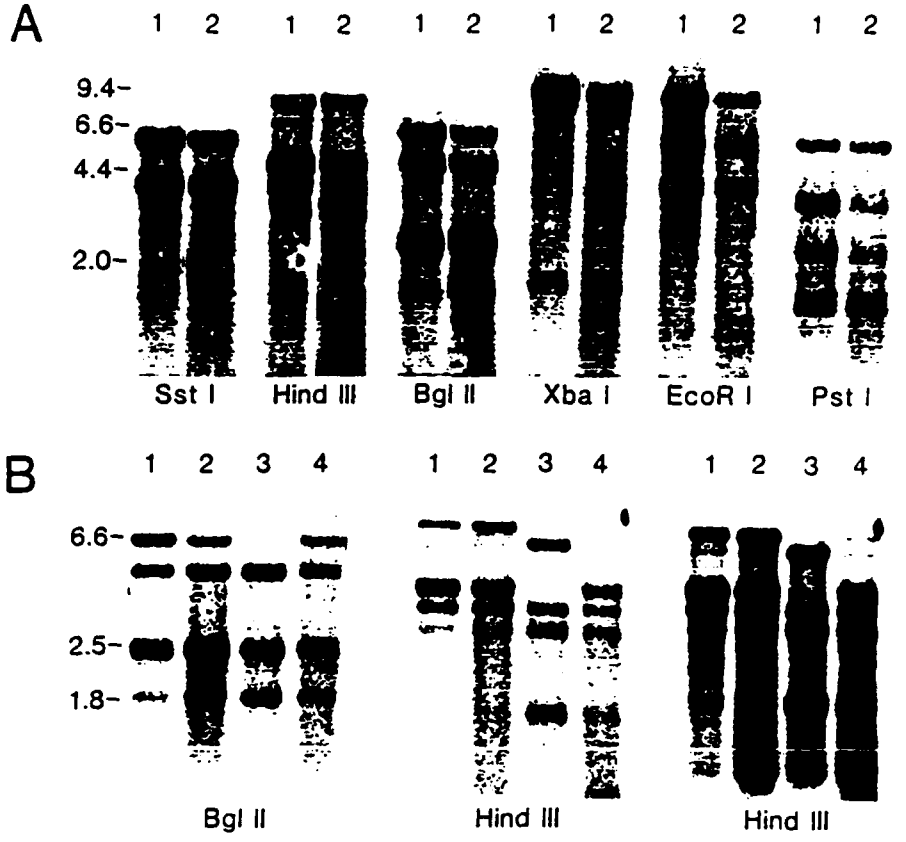
Virus	Pattern	Clone	Total
MNF1	E S X B U H P K M C A		Number
a	1 1 1 1 1 1 1 1 1 1 1	5, 9, 15, 16, 18	5
b	1 2 1 1 1 1 1 1 1 1 1	4, 14, 19	3
c	1 2 1 2 1 1 1 1 1 1 1	11, 13	2
d	1 1 1 2 1 1 1 1 1 1 1	10	1
e	1 1 2 2 1 1 1 1 1 1 1	1	1
f	1 1 2 2 1 1 1 1 1 1 2	12	1
g	1 1 2 1 1 1 1 2 1 1 1	2	1
h	1 2 2 2 1 1 1 1 1 1 2	6	1
i	1 2 1 3 1 1 1 1 1 1 1	3	1
j	1 2 1 4 2 1 1 1 1 2 2	17	1



Virus	Pattern	Clone	Total
MNF3	E S X B U H P K M C A		Number
a	1 1 1 1 1 1 1 1 1 1 1	1, 110, 111	3
b	1 1 1 1 1 1 1 2 1 1 1	103, 107	2
c	1 1 1 1 1 1 2 1 1 1 1	3, 108	2
d	1 1 1 1 2 1 1 1 1 1 1	10, 104	2
e	1 1 1 1 2 1 2 1 1 1 1	102	1
f	1 1 1 2 1 1 1 1 1 1 1	11	1
g	1 1 1 2 1 1 2 1 1 1 1	101	1
h	1 1 2 1 2 1 2 1 1 1 1	106	1
i	1 1 1 2 1 1 1 2 1 2 1	9	1
j	1 1 1 3 1 1 1 1 1 1 1	7	1
k	1 1 1 4 1 1 1 2 1 1 1	12	1
l	1 1 1 1 1 1 2 3 1 1 1	105	1
m	1 1 1 1 3 1 1 4 1 2 1	109	1

Figure 2. Southern blot restriction enzyme cleavage patterns of parallel and sequential isolates of HIV-1 (Panels A and B) and of cultured versus uncultured virus (Panel C). Panel A, lanes 1 and 2 represent viral isolates obtained by co-cultivating a single (split) sample of PBMC from an HIV-1 infected subject (RJS) with PHA-stimulated lymphocytes from two different, HLA-unrelated normal donors. Panel B, lanes 1 and 2, are the same as lanes 1 and 2 from panel A. Lanes 3 and 4 correspond to virus isolates derived from the PBMC of this same HIV-1 infected subject taken 2 weeks and 4 weeks after the first phlebotomy and co-cultured again with PBMC from two other unrelated normal donors. The right-hand blot in panel B is identical to the center blot except that the exposure time was lengthened from two to five days in order to identify faint signals. Notice that the predominant HindIII patterns of isolates 3 and 4 are distinguishable from isolates 1 and 2, yet with long exposure films persistence of common viral genotypes can be identified in all isolates. Panel C, Southern blot-hybridization patterns of HIV-1 DNA extracted directly from uncultured brain tissue (lanes 2) and from HIV-1 viral cultures derived by lymphocyte co-cultivation with the same brain tissue specimen (lanes 4). Lanes 1 show an HIV-1 positive control DNA from an unrelated virus isolate and lanes 3 represent uninfected negative control DNA.

Methods: Virus isolations were made by co-cultivation of patient peripheral blood mononuclear cells (PBMC) or brain tissue with PHA-stimulated normal donor PBMC as described[8]. The HIV-1 isolate from brain was subsequently transmitted to H9 cells and analyzed as shown (Panel C). Southern blot-hybridizations were performed using a near full-length (9kb) HIV-1 probe (pBH-10i) as described[8]. Autoradiogram exposure times were for 2 - 5 days.


A
1 2 1 2 1 2 1 2 1 2 1 2
9.4-
6.6-
4.4-
2.0-
Sst I
Hind III
Bgl II
Xba I
EcoR I
Pst I
B
1 2 3 4 1 2 3 4 1 2 3 4
6.6-
2.5-
1.8-
Bgl II
Hind III
Hind III

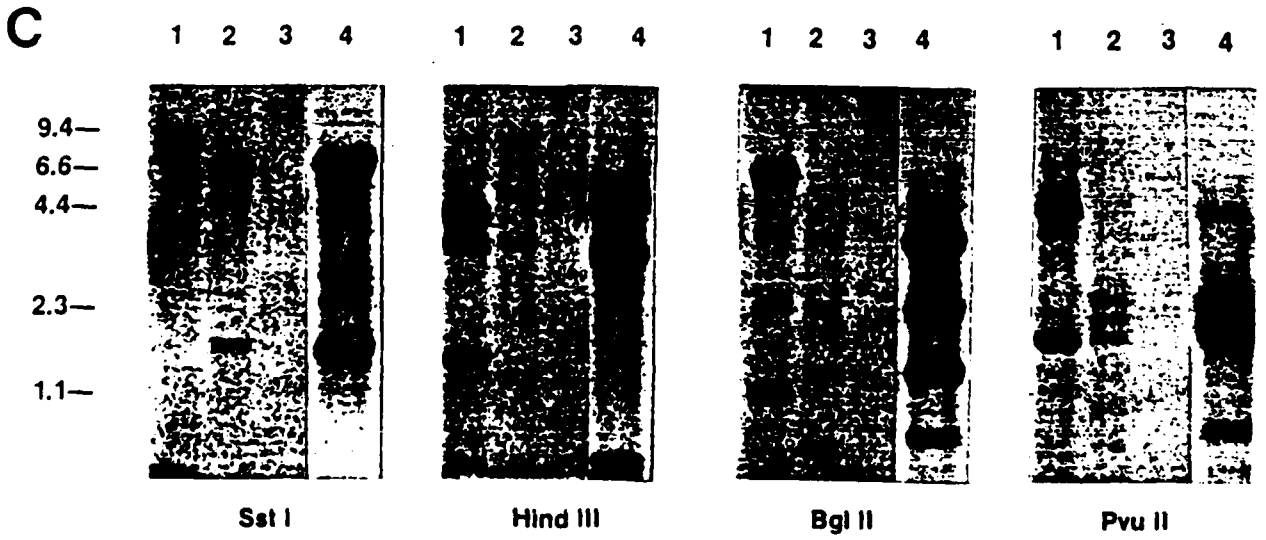

C
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
9.4—
6.6—
4.4—
2.3—
1.1—
Sst I
Hind III
Bgl II
Pvu II

Table I. Analysis of Variation Among Viral DNA Clones of HIV-1. Each of the 45 different viral clone patterns depicted in Figure 1 was compared pairwise to every other clone pattern and the percentage difference in restriction endonuclease cleavage sites between each pair was calculated as follows:

$$\frac{A + B}{C} \times 100 = \% \text{ Restriction Site Differences}$$

where, A equals the number of restriction sites present in one clone (X) that are missing in the other clone (Y); B equals the number of restriction sites present in clone Y that are missing in clone X; C equals the total number of restriction sites present in clones X and Y combined, with identical sites in the pair counted only once. For example, if clone X and clone Y had 25 restriction sites in common and no additional sites, they would have:

$$\frac{0 + 0}{25} \times 100 = 0\% \text{ Restriction Site Differences}$$

If clones X and Y each had 25 restriction sites but none were in common, they would have:

$$\frac{25 + 25}{50} \times 100 = 100\% \text{ Restriction Site Differences}$$

This analytic approach allowed each of the 45 distinct clone patterns to be compared with every other clone. Because EcoRI and SstI enzymes were used to clone the RJS and WMF viruses, respectively, these enzyme sites were not included in the analysis of variation among the RJS4, WMF1, and WMF3 clones. Because the two terminal SstI sites in all clones except WMF1b, c, h, i, and j actually represent redundant sequences in their LTRs, only one of the two was included in the analysis of variation. Thus, the first pair of clones compared in Table 1 (HXB2 versus LAV-Mal) differ by a total of 27 out of 43 restriction sites, or 63%.

$$\frac{17 + 10}{43} \times 100 = 63\% \text{ Restriction Site Differences}$$

Boxed in solid lines are the difference scores for viral clones derived from the RJS4, WMF1, and WMF3 isolates. The dashed lines highlight the comparison of viral clones between isolates WMF1 and WMF3, both of which were derived from the same chronically infected individual 16 months apart. In order to compare statistically the extent of dissimilarity among clones from within individual isolates (RJS4a-q; WMF1a-j; WMF3a-m) versus clones from independent (unrelated) isolates (HXB2, LAV-Mal, LAV-Eli, ARV2, WMJ1), the individual data points, not mean percentages, of the respective groups were compared by chi square analysis (see text).

	HXB2	LAV-MAL	LAV-ELI	ARV2	WMJ1	RJS4 a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	WMF1 a	b	c	d	e	f	g	h	i	j	k	l	m	WMF3 a	b	c	d	e	f	g	h	i	j	k	l	m
HXB2	—	63	62	41	49	46	45	45	44	45	47	49	46	45	42	43	45	41	45	45	51	58	46	45	42	44	45	46	51	45	46	49	54	55	53	51	50	53	51	51	56	51	50	55	63
LAV-MAL	63	—	58	68	63	61	63	59	62	59	66	63	64	63	61	63	66	63	63	63	59	66	57	59	61	58	59	60	62	63	61	66	57	58	56	58	57	56	54	58	59	58	57	58	64
LAV-ELI	62	58	—	70	58	67	66	69	68	65	65	66	67	68	70	66	65	65	62	69	66	65	59	58	59	61	62	63	64	62	59	65	59	61	62	61	63	58	61	64	58	62	59	65	59
ARV2	41	68	70	—	55	49	47	47	46	48	50	53	49	48	45	45	48	44	51	51	41	54	53	51	49	50	51	49	54	49	54	48	56	57	55	54	53	55	54	54	54	59	53	58	59
WMJ1	49	63	58	55	—	49	47	47	46	47	50	51	49	51	49	49	49	47	51	55	47	54	49	47	49	50	51	53	54	51	49	51	53	54	51	54	53	51	50	54	55	54	53	54	59
RJS4 a	46	61	67	49	49	—	11	04	11	07	15	04	15	07	07	10	14	07	12	18	08	15	36	34	36	38	40	42	43	40	36	49	41	43	44	38	41	39	42	43	44	43	41	47	53
b	45	63	66	47	47	11	—	08	07	11	04	15	11	17	11	14	17	11	08	18	22	19	39	38	39	41	43	44	46	43	39	51	44	46	42	41	39	42	41	41	47	46	44	45	56
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d	44	62	68	46	46	11	07	11	—	04	11	14	04	10	11	07	10	10	14	14	21	18	38	36	38	40	42	43	44	42	38	50	43	44	41	40	38	41	39	40	46	44	43	44	54
e	45	59	65	48	47	07	11	11	04	—	14	11	07	07	14	03	07	07	11	17	17	14	35	33	35	37	38	41	42	39	35	47	40	42	43	37	40	38	41	42	43	42	40	46	51
f	47	66	65	50	50	15	04	12	11	14	—	12	08	21	15	17	14	14	12	19	25	22	42	41	42	44	46	47	49	46	42	54	47	49	45	44	42	45	44	44	45	49	47	48	55
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k	43	63	66	45	49	10	14	14	07	03	17	14	10	03	10	—	03	03	14	14	14	17	37	35	32	34	36	38	43	36	37	45	42	43	44	39	42	40	43	43	44	39	37	47	53
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WMF1 a	46	57	59	53	49	36	39	39	38	35	42	39	41	40	41	37	40	35	39	44	39	42	—	04	07	04	07	10	11	14	07	31	14	17	18	11	14	11	15	17	18	17	14	21	29
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f	46	60	63	49	53	42	44	44	43	41	47	44	46	41	42	38	41	36	44	44	40	47	10	14	10	07	03	—	07	03	16	26	23	25	26	19	23	20	23	19	27	19	17	29	35
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j	49	66	65	48	51	49	51	51	50	47	54	51	53	45	49	45	47	43	51	51	47	53	31	29	25	28	30	26	33	24	30	—	31	33	35	33	35	29	32	38	34	28	31	38	44
WMF3 a	54	57	59	56	53	41	44	44	43	40	47	44	46	44	46	42	44	40	44	49	44	47	14	18	21	17	20	23	23	26	21	31	—	04	04	04	07	04	08	11	11	11	14	08	15
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e	50	57	63	53	53	41	39	39	38	40	42	44	41	44	41	42	44	40	44	44	44	47	14	18	21	17	20	23	23	26	21	35	07	11	04	04	—	11	08	04	18	17	21	07	22
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h	51	58	64	54	54	43	41	41	40	42	44	46	43	46	43	43	46	42	46	46	46	49	17	21	23	20	17	19	20	23	23	38	11	14	07	07	04	14	11	—	21	20	23	11	25
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k	50	57	59	53	53	41	44	44	43	40	47	44	46	40	41	37	40	35	44	44	39	47	14	18	14	11	14	17	23	20	21	31	14	11	18	17	21	11	15	23	11	11	—	21	29
l	55	58	65	58	54	47	45	45	44	46	48	50	47	50	47	47	50	46	50	50	50	53	21	25	28	24	27	29	30	32	28	38	08	11	04	11	07	12	08	11	19	18	21	—	16
m	63	64	59	59	69	53	56	56	54	51	55	52	53	56	57	53	51	51	56	56	52	59	29	32	34	31	33	35	37	39	34	44	15	19	19	19	22	19	23	25	19	25	29	16	—

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